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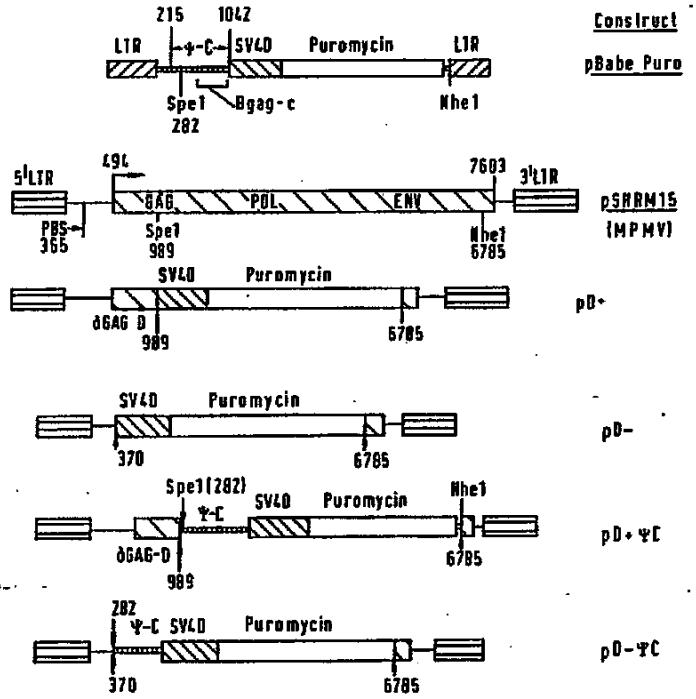

(57) Abstract

Retroviral vectors are based on a packaging sequence of a D-type retrovirus and are useful as heterologous gene delivery systems in gene therapy. D-type retrovirus do not share the oncogenic characteristics of the more conventionally used C-type retroviruses, and have a high packaging efficiency.

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D - TYPE RETROVIRAL VECTORS, BASED ON MPMV.

This invention relates to retroviral vectors. In particular, the invention relates to retroviral vectors which have reduced oncogenic risk, which is particularly relevant when used in the field of gene therapy, and packaging efficiencies comparable to natural viruses.

There has been much recent activity towards the idea of correcting genetic disease in humans or, in principle at least, in other animals by "gene therapy". As various review articles have pointed out (Weatherall *Nature* **349** 275-6 (1991); Miller *Blood* **76**(2) 271-8 (1990); Friedmann *Science* **244** 1275-81 (1989) and Anderson *Science* **226** 401-9 (1984)), progress has been slower than the amount of activity might suggest. One of the problems has been to devise a competent, safe and reliable method for introducing heterologous genetic material into the genome of target cells.

The use of retroviruses as vehicles for gene transfer is a promising approach. Unlike all other viruses, retroviruses replicate their RNA genome via a DNA intermediate that becomes permanently integrated into host cell DNA. Retroviruses are widespread in nature, and the many hundreds of isolates can be classified into groups according to biological properties, morphology and genome structure. Four types of particles, A, B, C and D, have been identified by electron microscopy. Most of the cancer-causing viruses that have been studied extensively are C-type oncoviruses, although the important mouse mammary tumour viruses have the B-type morphology. The C-type virus group itself consists of a vast array of viruses.

C-type oncoviruses have been the centre of much extensive study both biologically and molecularly. This has resulted in the development of several generations of C-type retroviral vector sand packaging cell lines, derived from both murine and avian viruses (Cosset *et al*, *J. Virol.* **64** 1070-1078 (1990) and Danos and Mulligan *Proc. Natl. Acad. Sci. USA*, **85**, 6460-6460 (1988)). These vectors generate recombinant retroviruses capable of delivering foreign genes into cells more efficiently than other physical means of gene transfer. Generation of these vectors has required the definition of the *cis*- and *trans*- acting functions required for the production of recombinant retrovirus particles (Miller *et al*, *Somatic Cell. Mol. Genet.* **12**, 175-183 (1986)). In particular, the most basic vectors must include the two viral long terminal repeats (LTRs), sequences required for reverse transcription (which lie both within and adjacent to the 5′ and 3′ LTRs), and an RNA packaging sequence which is required for encapsidation of viral genomes into viral particles (Swanstrom and Vogt, *Current Topics in Microbiology and Immunology*, **157** (1990)). The viral packaging sequence is required for the dimerisation of the two genomic length RNA molecules (Bieth *et al*, *Nucleic Acids Res.* **18** 119-127 (1990) and Prats *et al*, *J. Virol.* **64**, 774-783 (1990)) which are subsequently directed into the maturing diploid viral particle following binding by a *gag*-encoded zinc-finger protein (Leis and Jentoft *J. Virol.* **48**, 361-369 (1983)). In the case of the C-type murine leukaemia viruses (MLV), the most effective packaging sequence lies just downstream of the region containing both the 5′ splice donor site and the binding site for the tRNA primer (PBS) (which is used in reverse transcription for the initiation of minus strand DNA synthesis), and also includes a portion of the

gag gene (Bender *et al*, *J. Virol.* **61**, 1639-1646 (1987)). Packaging sequences have also been defined for the Human Immunodeficiency virus Type 1 (Lever *et al*, *J.Virol.* **62**, 4085-4087 (1989)) (Watanabe and Temin *Proc. Natl. Acad. Sci. USA*, **79**, 5986-5990 (1982)) and Avian Leukosis Virus (ALV) (Watanabe and Temin *Proc. Natl. Acad. Sci. USA*, **79**, 5986-5990 (1982)).

C-type vectors have proved of great value in defining aspects of the retroviral life cycle (Panganiban and Fiore *Science* **241**, 1064-1069 (1988)), in the identification of retroviral receptors (Albritton *et al*, *Cell* **57**, 659-666 (1989), O'Hara *et al*, *Cell Growth and Differentiation*, **1** 119-127 (1990) and Takeuchi *et al*, *J. Virol.* 1219-1222 (1992)), and even in approaches to gene therapy of human disease (Rosenberg *et al*, *N. Engl. J. Med.* **323** 570-578 (1990)). However, various limitations of C-type retroviral vectors have become apparent, especially in the field of gene therapy, partly because of the continual risk associated with these potentially oncogenic vectors (Temin H.M., *Science* **246**, 983 (1989)). Further, the host range for target cells is somewhat limiting: in particular, vectors more effective at infecting stem cell populations would be useful. Therefore, a different type of viral vector (retroviral or otherwise) is sought as an alternative to C-type vectors in gene transfer protocols.

It has now been realised that D-type retroviruses may form the basis of highly efficient vectors which do not suffer from all the disadvantages of those based on C-type retroviruses. This realisation is based on the discovery of a packaging sequence for D-type retroviruses.

Mason-Pfizer monkey virus (MPMV) is the prototype D-type retrovirus of the oncovirus family of retroviruses. The first isolate was derived from a female rhesus monkey (*M. mulatta*) with a breast carcinoma (Chopra and Mason, *Cancer Res.* **30**, 2081-2086 (1970)) and MPMV has now been molecularly cloned and sequenced (Sonigo *et al.*, *Cell* **45** 375-385 (1986)). Subsequent studies have failed to support any further link between this class of retroviruses and cancer. MPMV infection is, however, associated both with a T cell immunosuppression in young rhesus monkeys (Fine *et al*, *Natl. Cancer Inst.* **54**, 651-658 (1975)) and with the simian acquired immunodeficiency syndrome (SAIDS) in adult rhesus monkeys (Stromberg *et al*, *Science* **224** 289-292 (1984)). A second D-type retrovirus serotype was isolated from a Formosan rock macaque (*M. cyclopis*) also suffering from SAIDS (Marx *et al*, *Science* **223**, 1083-1086 (1984)), and subsequently other SAIDS retrovirus serotypes (SRV) have been isolated (Marx *et al*, *Science* **223** 1083-1086 (1984), Daniel *et al*, *Science* **223** 602-605 (1984), Marx *et al*, *J. Virol* **56** 571-578 (1985) and Sommerfelt *et al*, *J. Virol.* **64** 6214-6220 (1990)), including SRV-5. D-type retroviral infection in humans has not yet been linked with any disease.

According to a first aspect of the present invention, there is provided a recombinant or isolated RNA comprising a D-type retroviral packaging sequence or a recombinant or isolated DNA corresponding to or complimentary to such an RNA.

The D-type retroviral packaging sequence may be identical to a natural sequence, which may be isolated from a D-type retrovirus. An example of a D-type retrovirus is

Mason-Pfizer monkey virus (MPMV), the packaging sequence of which extends downstream of the $tRNA^{lys}$ primer binding site (PBS^{lys}), at position 365 of the MPMV genome (using the numbering system of pSHRM15, an infectious molecular clone of MPMV (Sonigo *et al.*, *Cell* 45 375-385 (1986) and Rhee *et al.*, *J. Virol.* 64 3844-3852 (1990))). It has been established, in the accomplishment of the present invention, that the 619 bases downstream of the PBS^{lys} are sufficient to confer packaging activity; however, it is possible that not all that sequence is necessary, and so not all of it need be present.

Fine detail mapping of the sequence, and site-directed mutagenesis, will further delineate the precise 5′ and 3′ boundaries of the natural D-type packaging sequence. It would be predicted that the 5′ extent of this sequence will lie downstream of the 5′ splice donor site of MPMV, so that only full length genomic transcripts, rather than spliced sub-genomic RNA molecules, will be efficiently packaged. A putative splice donor signal (5′-AGGT-3′) lies, upstream of the PBS^{lys}, at positions 312-315. Since any vector must retain the PBS for reverse transcription to occur (Swanstrom and Vogt, *Current Topics in Microbiology and Immunology*, **157** (1990), this sequence element effectively, therefore, represents the 5′ boundary of the packaging sequence. With respect to the 3′ boundary, however, further deletion analysis will be required to define the minimal natural 'core' sequence which is both necessary and sufficient for viral packaging.

As with all the features of this invention, the above sequence may be, and preferably is, identical to a natural sequence. However, it will be appreciated that

sequences which are not identical to natural sequences may work perfectly adequately and may even in some cases be better; for this reason their use is not ruled out. The invention therefore encompasses sequences which have qualitatively the same (relevant) function as a natural sequence but which have different base compositions. There will usually be homology between natural sequences and other sequences having qualitatively the same function; this homology may be at least 80% or even at least 90%, 95% or 99%, in increasing order of preference.

Alternatively or in addition, sequences useful in the invention may hybridise to a natural sequence whose function is sought. Hybridisation may be under stringent conditions (see Maniatis *et al.*, "Molecular Cloning: A laboratory Manual", Cold Spring Harbor Laboratory (1982), pp 387-389). An example of stringent hybridisation conditions is hybridisation at 4xSSC at 65°C, followed by washing in 0.1xSSC at 65°C for one hour. An alternative exemplary hybridisation condition is 50% formamide, 4xSSC at 42°C.

If hybridisation is not under stringent conditions, it may be under relaxed conditions. Examples of such non-stringent hybridisation conditions are 4xSSC at 50°C or hybridisation with 30-40% formamide at 42°C.

The D-type retroviral packaging sequence is useful in the construction of viral vectors. According to a second aspect of the invention, there is provided a retroviral vector, or a DNA construct having a strand corresponding or complementary to a retroviral vector, comprising: (a) sequence capable of providing retroviral long terminal repeats (LTRs); (b) sequence required for reverse

transcription; and (c) a D-type retroviral packaging sequence.

Long terminal repeats (LTRs) are present on double stranded DNA reverse-transcribed from retroviral genome RNA. A DNA construct in accordance with the invention will include the LTRs necessary for host cell genome incorporation and expression. The LTRs are not present as such in retroviral genomic RNA; instead, the genomic RNA contains sequences from a combination of which the LTRs are derived. Specifically, each LTR (which is usually in the order of 300 to 1000 long) is generally derived from a combination of sequences present at the 3' end (U_3), the 5' end (U_5) or both ends (R) of the RNA genome and has the structure U_3-R-U_5. Following synthesis, the viral DNA is integrated into cellular DNA so that the ends of the LTRs are directly joined to cellular sequences to form a stable structure, the provirus.

In the present invention, the LTR sequences will generally be derived from one or more D-type retroviruses such as MPMV.

Secondly, vectors in accordance with the invention also comprise sequence necessary for reverse transcription. Such sequence does not necessarily include sequence coding for the enzyme reverse transcriptase. Rather, retroviral provirus DNA contains a replication initiation site, in fact generally one on each strand of the double stranded DNA molecule. On the minus strand, the replication initiation site is designated r^- and is the tRNA binding site (RBS); on the plus strand, the replication initiation site is designated r^+ and is otherwise known as the purine-rich site.

In the present invention, the sequence necessary for reverse transcription will generally be derived from one or more D-type retroviruses such as MPMV.

Thirdly, vectors in accordance with the invention comprise a D-type retroviral packaging sequence, as described above.

Donor (D) and acceptor (A) splice sites may also be present. D and A sites are used naturally *in vivo* for expression of retroviral genes from spliced subgenomic RNA (mainly *env*). In retroviral vectors, such as provided by the present invention, they enable the construction of "double expression vectors" which can express two, rather than one, heterologous inserted nucleic acid sequence. As such, D and A sites are not themselves directly involved in retroviral packaging of the genomic RNAs.

These components of the vectors of the invention constitute the minimum needed for transcription and transmission of the viral genome. Such minimal vectors would not include sequences corresponding to the viral genes *gag* (coding for the group-specific (that is, viral core) antigens), *pol* (RNA-dependent DNA polymerase (reverse transcriptase)) or *env* (envelope proteins). In fact, the absence of one or more or even all three of such genes may be preferred, so that the indefinite production of infectious virus particles, once the proviral DNA in integrated with the host cellular DNA, is avoided.

D-type vectors defective or, ideally, completely deleted in *env* are particularly advantageous as there will be no

functional envelope gene transferred to target cells. This may reduce the risk of immunosuppression, the major pathogenic activity of the D-type retroviruses.

Vectors or constructs in accordance with the invention will often contain exogenous DNA (or RNA corresponding to it, as the case may be). For use in gene therapy, a vector in accordance with the invention will contain the exogenous gene or other DNA which it is desired to transfer to the intended recipient. Exogenous DNA may code for the replacement or substitute of a defective or missing enzyme or other protein in the patient (whether human or animal). The enzyme or other protein may normally function within a cell or circulate round the body; examples of circulating proteins include hormones and blood factors. Genes coding for proteins whose levels do not have to be controlled precisely and/or genes which can cause disease with a single defect are attractive candidates for the invention. Examples include:

Haemophilias
- Factor VIII deficiency
- Factor IX deficiency

Immunodeficiencies
- Adenoside deaminase deficiency
- Purine nucleoside phosphorylase deficiency

Urea cycle disorders
- Ornithine transcarbamylase deficiency
- Arginosuccinate synthetase deficiency

Anaemias
- β-thalassaemia
- Sickle cell anaemia

Emphysema
- α_1-antitrypsin deficiency

Lysosomal storage disorders
    Glucocerebrosidase deficiency
Other metabolic disorders
    Phenylalanine hydroxylase deficiency
    Hypoxanthine-guanine phosphoribosyl transferase deficiency
Cancers
    Cytokine genes
    Cytotoxic genes
    MMC genes
    Tumour suppressor genes
    Antisense genes

Exogenous DNA may include complementary, genomic or synthetic DNA coding for a protein of interest. In addition, regulatory sequences may be present. For example, DNA coding for a protein of interest may be under the control of a suitable promoter. In some embodiments of the invention, only control sequences may be present if the intention is, for example, to "rescue" the expression of genes present but not correctly functioning in the intended recipient.

The exogenous DNA may comprise a suitable marker DNA sequence; this may be primarily for investigative or experimental purposes, but a suitable selectable marker may enable infective particles containing retroviral vector RNA to be selected, as will be described below. Many selectable markers are based on antibiotic resistance; an example is puromycin resistance. Others include geneticin, L-histidinol, hygromycin B, gpt, thymidine kinase and/or APRT.

It is to be stressed that the nature of the exogenous DNA will depend entirely on the intended purpose of the retroviral vectors of the invention, and so is not particularly limited.

To be useful in delivering its genetic payload, whatever it may be, to a target cell, a retroviral vector must be rendered infective. This is usually achieved by means of a helper virus, using an appropriate packaging host cell. For example, a DNA construct in accordance with the invention is introduced into a packaging host cell which is a suitable host for the helper virus (such as HeLa for MPMV or SRV-5) by any suitable transfection method, such as calcium phosphate precipitation. Existing methods and materials known in the art may be used for these purposes; a useful summary can be found in "*Methods in Molecular Biology, Vol. 8: Practical Molecular Virology: Viral Vectors for Gene Expression*", particularly Chapter 1 ("The Retroviral Life Cycle and the Molecular Construction of Retrovirus Vectors", by Richard Vile) and Chapter 5 ("Selectable Markers for Eukaryotic Cells", also by Richard Vile). This book, particularly the chapters referred to above, and the references cited therein, are herein incorporated by reference.

Host cells transfected with DNA constructs as described above and infectious particles containing retroviral vectors as described above are also within the scope of the invention.

The viral particles would be used to infect target cells (ie. those usually which are defective in expression of the deviant gene or those which can act to secrete the relevant protein) either *ex vivo* followed by replacement

of the infected cells into the patient or, conceivably, *in vivo* directly.

Stem cells are the ideal target so that the "defective" cell population can be rendered non defective by permanent division and replenishment of converted cells from the altered stem cell pool. The problem for all cell lineages is to identify, infect and purify the relatively sparse stem cells from the total cell population. Stem cells do not have to be infected - ie, dividing cells of the mature lineage could be infected by the retroviruses. However, if stem cells are not infected continual, repeated therapy will be required (possibly) because the corrected cell population will be lost without replenishment from any "corrected" self-replicating stem cells.

The particles can be used as markers for infection both *in vivo* (ie. cell lineage determination etc) or *in vitro* (ie. receptor identification studies).

The invention will now be illustrated by the following Example. The Example refers to the accompanying drawing, in which:

FIGURE 1 shows the molecular synthesis of constructs A, B, C and D from pSHRM15 and pBabe Puro. The following points of notation apply:

Ψ-*C* refers to the extended packaging sequence of MoMLV (Bender *et al.*, 1987);

Numbering in A to D refers to the junction nucleotide of pSHRM15 preceding/succeeding sequences of pBabe Puro (Sonigo *et al.*, 1986);

Numbering in italics refers to nucleotide sequence of MoMLV (Weiss *et al.*, 1985);

∂GAG-D refers to the first 495bp of MPMV GAG gene;

∂GAG-c refers to the first 342bp of Mo-MLV GAG gene; and

PBS refers to the tRNA binding site of MPMV (Sonigo *et al.*, 1986)

EXAMPLE

The packaging sequence of MPMV was localised by constructing four separate recombinant proviral genomes (Figure 1). A, [p(∂gag^{D})$^{+}$], was generated by ligating an SV40-Puromycin selectable marker cassette between the *Spe*I (position 989) and *Nhe*I (position 675) restriction sites in pSHRM15, an infectious molecular clone of MPMV, (Sonigo *et al*, *Cell* 45 375-385 (1986) and Rhee *et al*, *J. Virol.* **64**, 3844-3852 (1990)). In principle, any infectious molecular clone of MPMV could be used. As the sequence of MPMV has been published (Sonigo *et al*, (loc. cit.), polymerase chain reaction (PCR) technology may be used to clone it. The selectable marker cassette was derived from the C-type retroviral vector, pBabe Puro (Morgernstern and Land *Nucleic Acids Res.* **18**, 3587-3596 (1990)), by PCR amplification in which artificial *Spe*I and *Nhe*I sites were incorporated into the 5′ and 3′ primers respectively. Hence, A retains 624 bp downstream from the tRNA^{lys} primer binding site (PBS^{lys}) (position 365). This includes 495 bp of the *gag* gene but none of *pol* and only a small part of *env* (818 bp). In construct B, [p(∂gag)$^{-}$], the SV40 puromycin cassette of pBabe Puro was amplified with primers which allowed it to be cloned into pSHRM15 at position 370, just downstream, but including all of, the PBS^{lys}. Therefore, B lacks 619 bp

of sequence retained in construct A. To generate C, [p(∂gag^{D})$^{+}$(Ψ^{C})], the *Spe*I-*Nhe*I fragment of pBabe Puro was cloned between the *Spe*I and *Nhe*I sites of pSHRM15. This fragment of pBabe Puro includes most of the extended packaging sequence (Ψ+) (760 bp out of 827 bp) of the C-type Moloney Murine leukaemia virus (Mo-MLV) (14, 24), as well as the SV40 puromycin cassette. Finally, for D, [p(∂gag)$^{-}$(Ψ^{C})], this same *Spe*I-*Nhe*I fragment of pBabe Puro was amplified using PCR primers permitting it to be cloned into the same site (position 370) as for construct B - that is just downstream of the PBS^{lys}.

All four of these D-type constructs, along with pBabe Puro, were transfected separately into HeLa cells, as well as into the Murine Leukaemia Virus amphotropic (MLV-A) and ecotropic (MLV-E) C-type packaging cell lines PA317 and Ψ2 (Miller and Buttimore *Mol. Cell Biol.* **6**, 2895-2902 (1986) and Mann *et al*, *Cell* **33** 153-159 (1983)) using the calcium phosphate precipitation technique (Chen and Okayama *Mol. Cell Biol.* **7** 2745-2752 (1987)). Transfected clones were selected in puromycin (sigma) at 1.25μg/ml and approximately 100 clones from each transfection were pooled and maintained in selection.

10^5 cells of each of the five populations of transfected HeLa cells, along with an untransfected control, were then mock infected or infected with either wild type MPMV or the D-type retrovirus (SRV-5 (Daniel *et al*, *Science* **223** 602-605 (1984)), using cell-free supernatants (0.45μm-filtered) from virus producing cells. After 10-14 days, syncytia were clearly observed exclusively in the infected cell populations. Cell-free supernatants were harvested and used to infect fresh HeLa or NIH 3T3 cells in the presence of polybrene (Aldrich) at 4μg/ml.

Two days after infection, the target cells were split into puromycin selection and three weeks later surviving colonies were counted. Similarly, cell free supernatants were harvested directly from 10^7 cells of the transfected/selected populations of PA317 and Ψ2 cells and used to infect target HeLa or NIH3T3 cells which were also then selected in puromycin. Any titre of puromycin resistant colonies would indicate that a vector genome had been rescued from HeLa, PA317 or Ψ2 cells lines by the relevant wild type virus (MPMV, SRV-5, MLV-A or MLV-E). For this to occur, packaging of the vector genomic length RNA transcripts into capsid particles, reverse transcription of the RNA into DNA and productive integration of the resulting DNA provirus into the target cell would all be required. The results of these rescue experiments are shown in Table 1.

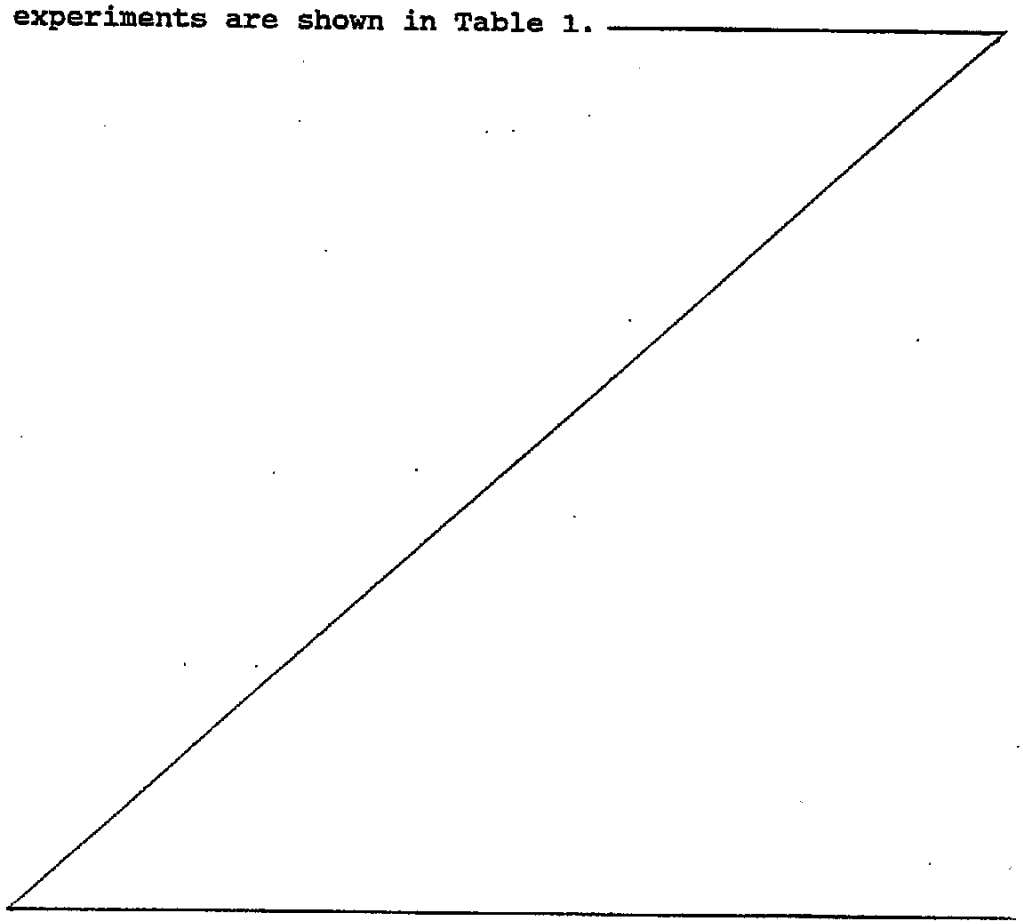


Table 1: Detection of recombinant MPMV or SRV-5 virus following rescue of vectors carrying a D-type packaging sequence.

Construct Transfected	Target Cell Transfected	Titre of Recombinant Virus Released Following Rescue by Wild Type Virus - Puromycin C.F.U./ml. (Titre of Wild Type MPMV co-released from transfected cells). (*syncytial inducing units/ml.*)							
		Rescuing Virus:							
		MPMV		SRV-5		MLV-A		MLV-E	
	Titering Cell:	HeLa	3T3	HeLa	3T3	HeLa	3T3	HeLa	3T3
None	HeLa	0 (10^4)	0	0	0	-	-	-	-
	PA317	-	-	-	-	0	0	-	-
	Ψ2	-	-	-	-	-	-	0	0
A	HeLa	$1.2x10^2$ (10^2)	0	80	0	-	-	-	-
	PA317	-	-	-	-	0	0	-	-
	Ψ12	-	-	-	-	-	-	0	0
B or D	HeLa	0 (10^3)	0	0	0	-	-	-	-
	PA317	-	-	-	-	0	0	-	-
	Ψ2	-	-	-	-	-	-	0	0
C	HeLa	$1.0x10^2$ (10^2)	0	80	0	-	-	-	-
	PA317	-	-	-	-	0	0	-	-
	Ψ2	-	-	-	-	-	-	0	0
pBabe Puro	HeLa	0 (10^3)	0	0	0	-	-	-	-
	PA317	-	-	-	-	$2.8x10^3$	$1x10^4$	-	-
	Ψ2	-	-	-	-	-	-	0	$2x10^5$

Titre values represent the average number of puromycin-resistant colonies produced/ml of cell-free supernatant, harvested from approximately 10^7 cells infected with ~10^3 virus particles of MPMV or SRV-5 10-14 days previously or from ~10^7 PA317 or Ψ2 cells transfected with the appropriate vector. Supernatant recovered from all the transfected cells lines gave no titre on either HeLa or 3T3 cells in the absence of infection with a wild type virus. Values shown are the mean of at least two separate experiments.

The symbol "-" means "not done".

Whereas both vectors A and C could be efficiently rescued from transfected HeLa cells by both D-type viruses, neither construct B nor D were rescued. None of the D-type constructs could be rescued by the C-type viruses MLV-A or MLV-E, although, as expected, the C-type vector pBabe Puro was packaged to high titre. Similarly, pBabe Puro could not be rescued by either d-type virus from HeLa cells.

To confirm that the virus rescued by MPMV infection of HeLa cells transfected by constructs A or C are genuinely MPMV recombinants, receptor interference and virus neutralisation studies were carried out. Supernatant from HeLa/A transfectants infected with MPMV was used to infect human Raji cells or Raji cells productively infected with MPMV or SRV-5. Whereas the Raji cells survived selection in puromycin following infection (at a titre of recombinant virus of 10^2 puromycin-resistant cfu/ml), no survivors were seen following infection of Raji/MPMV or Raji/SRV-5 with the recombinant stocks and selection in puromycin (Table 2).

Table 2: Cell tropism and demonstration of receptor interference of recombinant MPMV virus stocks.

Target Cell Line for Infection	Number of Puromycin-resistant colonies (/ml of virus supernatant).	
Source of supernatant:	HeLa/A rescued by MPMV	HeLa
Hela	1.3×10^2	0
NIH 3T3	0	0
Raji	1.0×10^2	0
Raji/MPMV	0	0
Raji/SRV-5	0	0

This demonstration of receptor interference (Sommerfelt and Weiss *Virology* **176** 58-69 (1990), Steck and Rubin *Virology* **29** 628-641 (1966) and Steck and Rubin *Virology* **29** 642-653 (1966)) reflects the inability of one virus to infect a cell which is already infected by a virus which uses the same cellular receptor. Therefore, the recombinant virus recovered from HeLa/A transfected cells recognises the same cellular receptor as both MMV and SRV-5 on Raji cells. It has already been demonstrated that these two D-type viruses utilise a common receptor (Sommerfelt and Weiss, *loc. cit.*). Furthermore, the recombinant viral stock was completely and specifically, neutralise by preincubation with anti-MPMV rabbit anti-serum, whereas a normal rabbit serum or normal culture medium were both unable to neutralise the recombinant virus when it was plated on HeLa cells (Table 3). The neutralising anti-serum was, however, unable to block infection of HeLa cells by the C-type virus released by PA317 cells transfected with p Babe Puro (Table 3).

Table 3: Neutralisation of recombinant MPMV Puromycin virus.

Supernatant	Treatment of supernatant prior to infection:	Titre of treated supernatant on HeLa cells:
HeLa/A transfectants rescued with MPMV	Rabbit anti-MNPMV serum*	0 (puro c.f.u./ml)
	Normal rabbit serum*	90 "
	Culture medium*	1.1×10^2 "
PA317/pBabe Puro transfectants	Rabbit anti-MPMV serum*	3×10^3 "
	Normal rabbit serum*	1×10^3 "
	Culture medium*	3×10^3 "

*Virus stocks were incubated with a 1:20 dilution of sera or culture medium alone for 60 minutes at 37°C and were then plated on HeLa cells.

Taken together, these data confirm that the recombinant virus rescued from HeLa/A transfectants have the envelope specificity of MPMV rather than of any endogenous retrovirus which might have been activate in the transfected HeLa cells.

Therefore, only recombinant D-type genomes which retained the 619 bp fragment of MPMV lying downstream of the PBS^{lys} could be rescued from transfected HeLa cells by D-type retroviruses (constructs A and C). When this sequence was absent (construct B) or replaced by 760 bp of sequence derived from the packaging signal of a C-type retrovirus, the vector was also no longer rescued by D-type viruses (construct D). The packeagable recombinant genomes (A and C) appear to be able to compete for packaging with wild type genomes since any titre of recombinants appeared to reduce the levels of wild type virus released from the producer cells (Table 1). When no recombinant genomes, or genomes that could not be rescued, were present in HeLa cells, the titre of wild type virus that could be generated from these producer cells was generally increased. This would suggest that the vectors A or C might be rescued to higher titres than those observed here if a helper-free D-type packaging cell line were used in which there are no (wild-type) genomes competing for packaging. Interestingly, SRV-5 was able to rescue both MPMV-derived constructs at similar levels to MPMV, suggesting that there is a high degree of cross-recognition of this putative packaging sequence within the D-type retroviruses. This

heterologous recognition between viruses of the same type probably occurs, therefore, at the level of interaction of the (*gag*-encoded) viral packaging protein (Leis and Jentoft *J. Virol.* **48**, 361-369 (1983)) with the secondary structure formed in the RNA dimer.

Previous work has shown that a range of C-type viruses can rescue a C-type vector from a human HOS cell line (Takeuchi *et al*, *Virology* **186** 792-294 (1992)) but the C-type vector could not be rescued by a D-type virus. The data in the present in invention confirm these findings since pBabe Puro could not be rescued by either MPMV or SERV-5 from the human HeLa cell line. It has now been shown that a similar, reciprocal relationship exists - that is that two D-type viruses can rescue a D-type vector but that C-type viruses cannot cross-rescue the vector (constructs A and C could not be packaged by PA317 or Ψ2 cells). Moreover, the presence of most of the extended C-type Ψ packaging sequence (Bender *et al*, *J. Virol.* **61**, 1639-1646 (1987)) was insufficient to allow rescue of an otherwise D-type vector by MPMV or SRV-5 (construct D). This would suggest that a stringent selection operates in viral packaging at the level of recognition of the secondary structure formed by the RNA packaging sequence with heterologous viral capsid proteins (Leis & Jentoft, *loc. cit.* and Takeuchi *et al.*, *loc. cit.*). Additionally, rescue of a recombinant genome by wild type virus requires compatibility of the *cis*-acting genome sequences with the viral structural and enzymatic proteins for all stages in the viral life cycle, not just at the stage of viral packaging. This is shown here by the inability of the C-type packaging sequence (either alone, or in tandem with a D-type sequence) to allow rescue of an otherwise D-type genome

by wild type C-type (Constructs C and D). Presumably, the C-type reverse transcriptase- and integrase-associated proteins are unable to recognise the D-type genomic sequences in the vectors. Therefore, by analogy with the position of the Ψ sequence in other retroviruses (Bender *loc. cit.*, Lever *et al*, *J.Virol.* **62**, 4085-4087 (1989) and Watanabe and Temin *Proc. Natl. Acad. Sci. USA*, **79**, 5986-5990 (1982)) the results shown here suggest that the 619 bp fragment of MPMV identified here as necessary for efficient rescue represents a generalised D-type retroviral packaging sequence. (A role for this sequence in D-type specific reverse transcription and/or integration cannot, however, be excluded at this stage.

CLAIMS

1. A recombinant or isolated RNA comprising a D-type retroviral packaging sequence or a recombinant or isolated DNA corresponding to or complimentary to such an RNA.

2. An RNA or DNA as claimed in claim 1, wherein the D-type retroviral packaging sequence is identical to a natural sequence.

3. An RNA or DNA as claimed in claim 2, wherein the D-type retroviral packaging sequence is derived from Mason-Pfizer monkey virus (MPMV).

4. An RNA or DNA as claimed in claim 3, wherein the packaging sequence comprises sequence corresponding to the 619 bases downstream of the $tRNA^{lys}$ primer binding site (PBS^{lys}), at position 365 of the MPMV genome (using the numbering system of pSHRM15).

5. A retroviral vector, or a DNA construct having a strand corresponding or complementary to a retroviral vector, comprising: (a) sequence capable of providing retroviral long terminal repeats (LTRs); (b) sequence required for reverse transcription; and (c) a D-type retroviral packaging sequence.

6. A vector or construct as claimed in claim 5, wherein the sequence capable of providing LTRs is derived from a D-type retrovirus.

7. A vector or construct as claimed in claim 5 or 6, wherein the sequence required for reverse transcription

comprises a replication initiation site derived from a D-type retrovirus.

8. A vector or construct as claimed in claim 5, 6 or 7, comprising retroviral donor (D) and acceptor (A) splice sites.

9. A vector or construct as claimed in any one of claims 5 to 8 which is defective or deleted in *env*.

10. A vector or construct as claimed in any one of claims 5 to 9 which is defective or deleted in *gag*.

11. A vector or construct as claimed in any one of claims 5 to 10 which is defective or deleted in *pol*.

12. A vector or construct as claimed in any one of claims 5 to 11, comprising exogenous nucleic acid.

13. A vector or construct as claimed in claim 12, wherein the exogenous nucleic acid codes for a protein.

14. A vector or construct as claimed in claim 13, wherein the protein is, or has qualitatively the same activity as, factor VIII, factor IX, adenoside deaminase, purine nucleoside phosphorylase, ornithine transcarbamylase, arginosuccinate synthetase, haemoglobin, α_1-antitrypsin, glucocerebrosidase, phenylalanine hydroxylase or hypoxanthine-guanine phosphoribosyl transferase.

15. A vector or construct as claimed in claim 12, wherein the exogenous nucleic acid has, or is capable or having, a regulatory function.

16. A vector or construct as claimed in any one of claims 12 to 16, wherein the exogenous DNA comprises a suitable marker sequence.

17. A vector or construct as claimed in claim 16, wherein the marker is a selectable marker.

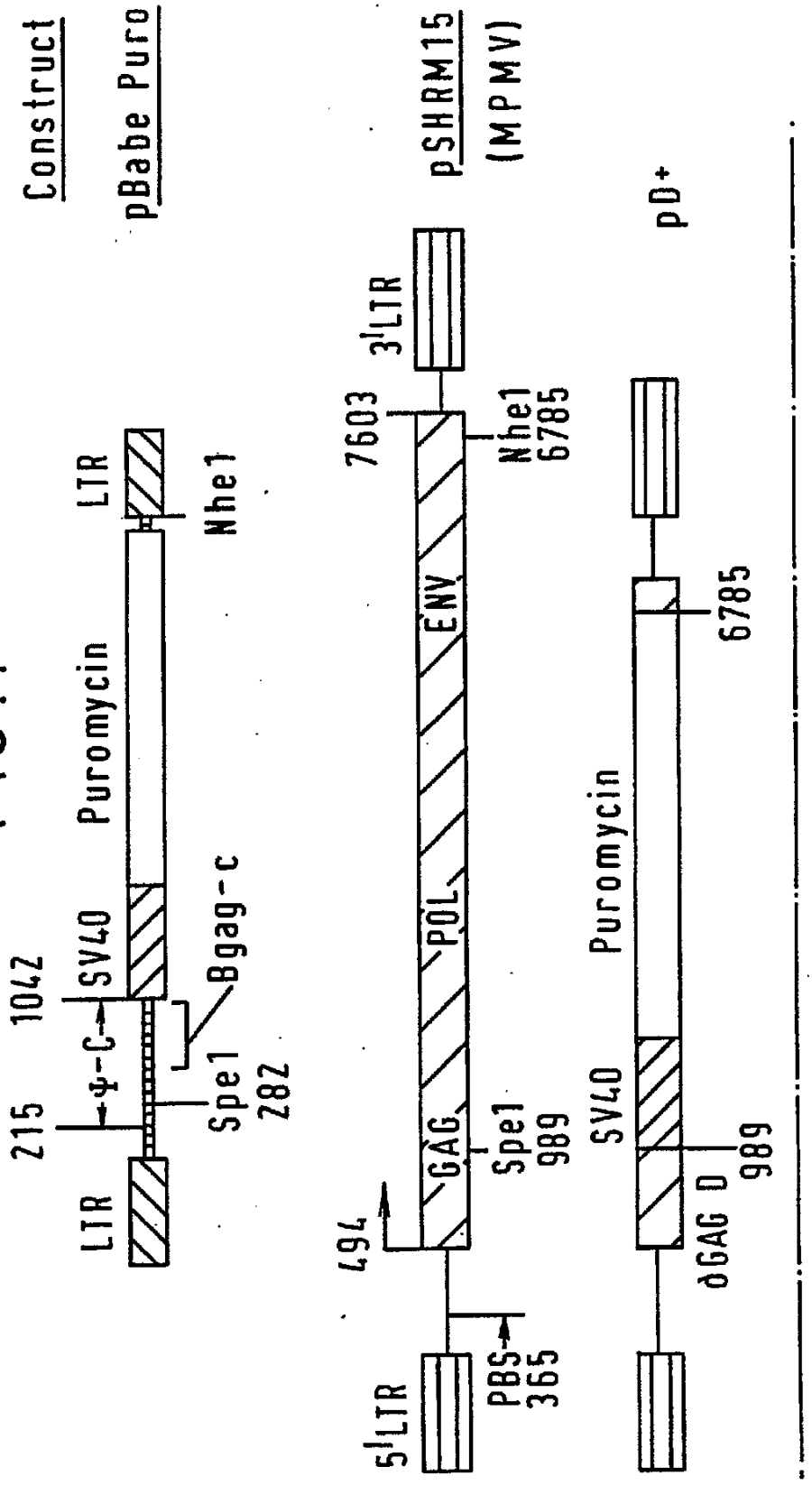
18. A vector or construct as claimed in claim 17, wherein the selectable marker is antibiotic resistance.

19. A host cell containing a vector or construct as claimed in any one of claims 5 to 18

20. An infectious particle comprising a vector as claimed in any one of claims 5 to 18.

21. An infectious particle as claimed in claim 20 for use in medicine, particularly gene therapy.

FIG. 1


Construct
pBabe Puro
LTR
Nhe1
Puromycin
SV40
1042
Ψ-C
215
Bgag-c
Spe1
282
LTR
pSHRM15
(MPMV)
3'LTR
7603
Nhe1
6785
ENV
POL
GAG
Spe1
989
494
5'LTR
PBS
365
pD+
6785
Puromycin
SV40
989
ΔGAG D

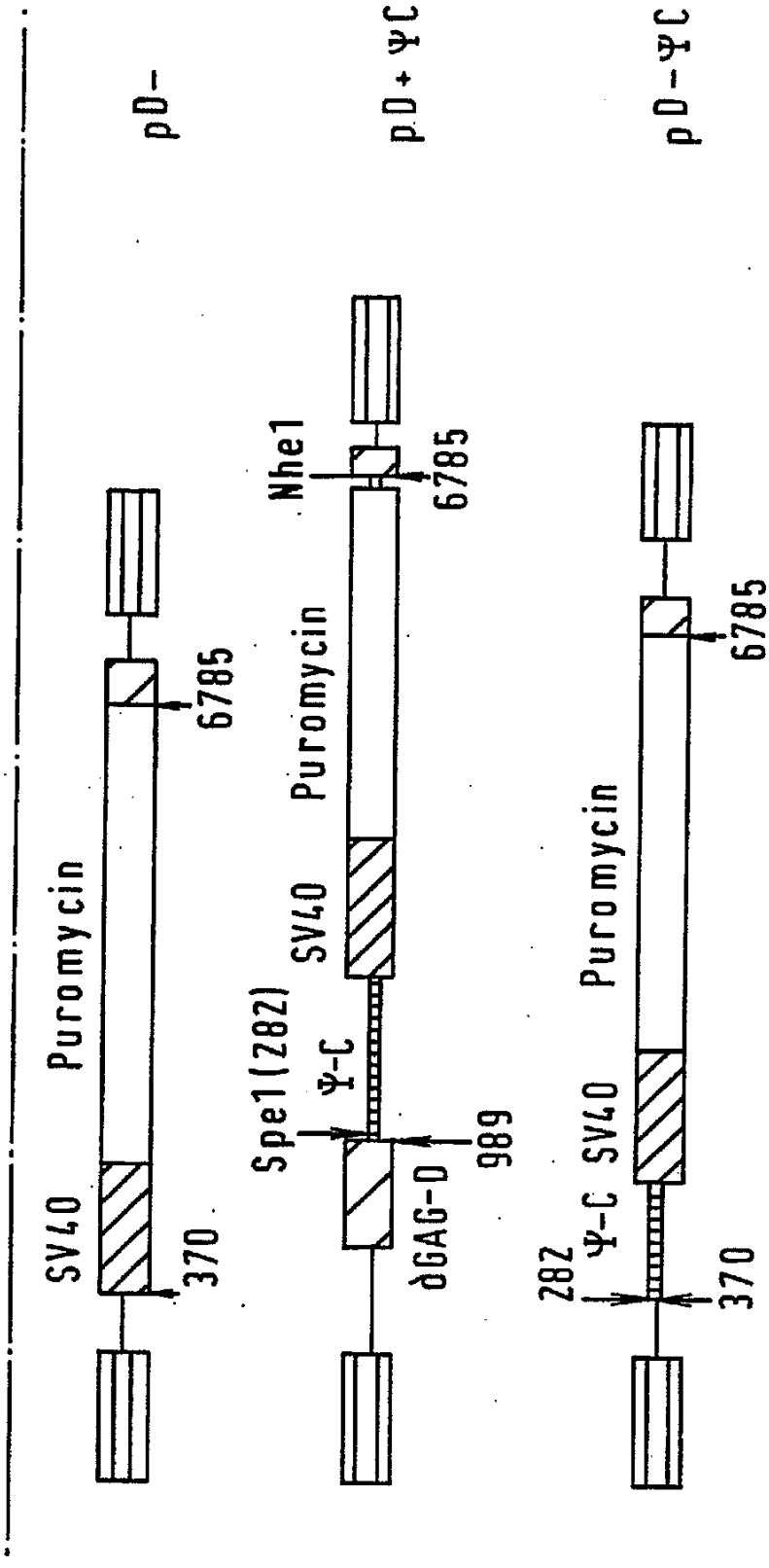

pD-
pD+ΨC
pD-ΨC
Nhe1
6785
Puromycin
SV40
370
Spe1(282)
Ψ-C
dGAG-D
989
282

FIG. 1 (CONTD.)

ERNATIONAL SEARCH REPORT

nformation on patent family members

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9317118	02-09-93	NONE	